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Introduction

Appropriate fetal growth and development relies upon the placenta to provide communication between fetal and maternal circulation to ensure the adequate delivery of nutrients and gasses, immune protection, and defense from potentially harmful environmental exposures. Disruption of placental morphogenesis and function is associated with adverse outcomes including poor fetal growth, preterm birth, and pre-eclampsia, as well as the origins of adult diseases such as lung disease, metabolic syndrome, and cardiovascular disease.

Normal placental development is affected by maternal, fetal, and environmental factors. Despite well-documented adverse pregnancy outcomes such as preterm labor and low birth weight, maternal cigarette smoking continues to be a common prenatal exposure with approximately 10% of babies exposed *in utero* during the third trimester in the United States (PRAMS/CDC)[1]. Maternal cigarette smoke exposure (CSE) is the predominant risk factor for having an infant that is small for gestational age (SGA) and predisposes a child to diseases in childhood and later in life[2-3].

Cigarette smoke is a complex mixture of thousands of compounds with potentially hazardous consequences that not only directly affect the developing fetus, but also disrupt placenta development and function. Maternal CSE during pregnancy is associated with morphological changes in the full-term placenta that include altered intervillous spaces, reduced fetal capillary volume, and increased trophoblast volume attributed to reduced apoptosis and hyperplasia. The individual components of maternal cigarette smoke and the biological pathways which they perturb to adversely affect the developing baby and the placenta have not been fully explained.

Objective

Identify major biological pathways in human placenta that are altered in response to maternal cigarette smoke exposure that may mediate the effects on the developing baby and placental function.

Methods

Figure 1. Experimental Design

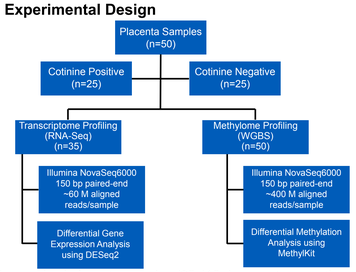
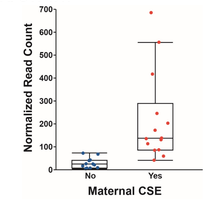


Figure 1. Anonymized placenta samples (85-115 days post-conception, n=50) were received from the Central Laboratory for Human Embryology (University of Washington, Seattle, WA). All tissues were flash frozen and maintained at -80ºC prior to use. The use of these tissues was declared non-human subjects research by the University of Missouri-Kansas City Pediatric Health Sciences Review Board. Maternal CSE was inferred by measuring the presence of cotinine in the placenta as previously described[4]. DNA and RNA isolated from human placenta samples were processed and analyzed as illustrated for RNA Sequencing (RNA-Seq) and whole-genome bisulfite sequencing (WGBS), respectively.

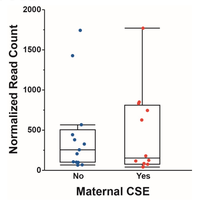
Results

Figure 2. Maternal CSE alters gene expression in the placenta

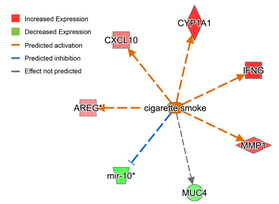
A. *CYP1A1*



B. *CGB*



C. Upstream Regulator: Cigarette Smoke



D. Expression of individual genes

Gene	Fold change	p-value	Adjusted p-value
CYP1A1	7.24	2.46E-12	9.08E-08
IFNG	5.88	0.033199	0.994428
MMP1	2.93	0.003874	0.994428
CXCL10	2.34	0.018266	0.994428
AREGB	2.03	0.019757	0.994428
Mir-10	0.37	0.003229	0.994428
MUC4	0.44	0.024442	0.994428

Figure 2. Transcripts for 204 genes were differentially expressed in response to maternal CSE (nominal p-value < 0.01). **A)** As expected, expression of *CYP1A1* was 7.3-fold higher in placenta samples positive for maternal CSE compared to those without (p=2.46E-12). CYP1A1 is a member of the Phase 1 drug-metabolizing enzyme family and substrates for CYP1A1 include components of cigarette smoke (benzo[a]pyrene, B[a]P) and estrogens. *CYP1A1* expression is known to be regulated by cigarette smoke exposure via interaction of B[a]P with the AHR transcription factor. **B)** Expression of *CGB* was also differentially expressed in samples positive for maternal CSE compared to those without (p=3.48E-04). *CGB* encodes for the β-subunit of human chorionic gonadotropin (hCG) and is integral to the establishment and maintenance of pregnancy. **C)** We performed Ingenuity Pathway Analysis (IPA, www.ingenuity.com) to explore major biology pathways associated with changes in gene expression. Genes with at least 2-fold change in expression and a p-value <0.05 were submitted for analysis. The upstream regulator "Cigarette Smoke" was associated with maternal CSE with an activation z-score of 2.365 (p-value of overlap 1.03E-3). **D)** Expression of individual genes in the Cigarette Smoke pathway.

Figure 3. DAVID pathway analysis for genes associated with DMRs

Category	Term	Count	%	p-value
GAD_DISEASE	Tobacco Use Disorder	177	32.96	2.31E-16
GAD_DISEASE	Echocardiography	40	7.45	5.44E-10
GAD_DISEASE	Body Mass Index	42	7.82	2.57E-08
GAD_DISEASE	Tuncia Media	22	4.10	1.93E-07
GAD_DISEASE	Cholesterol, HDL	37	6.89	4.05E-07
GAD_DISEASE	Hip	33	6.15	4.28E-07
GAD_DISEASE	Diabetes Mellitus	20	3.72	1.23E-06
GAD_DISEASE	Triglycerides	36	6.70	1.31E-06
GAD_DISEASE	Uric Acid	14	2.61	1.80E-06
GAD_DISEASE	Cholesterol, LDL	36	6.70	1.26E-05

Figure 3. WGBS identified 7558 differentially methylated regions (DMR), each containing ≥3 CpGs in a window of 200bp with the same direction of effect, of which 542/7558 DMRs contained ≥5 differentially methylated CpGs. We applied the Genomic Regions Enrichment Annotation Tools (GREAT) for the prediction of target genes for the identified DMRs. Among the many putative associated genes with altered methylation in regulatory elements upon CSE was *CYP1A2*, *CYP1B1* and *NRG1*, where the latter gene is known to promote extravillous trophoblast formation in placental explants. We performed pathway analysis by DAVID[7-8] for all genes (N=569) associated with DMR containing ≥5 differentially methylated CpGs and found Tobacco Use Disorder (N=177 genes, p=2.3xE-16) as the top associated disease category.

Figure 4. Known TFBS motifs enriched in DMRs

A. Top 10 enriched TFBS motifs found in differentially methylated CpG dinucleotides

Rank	Motif	Name	p-value	q-value (Benjamini)	% of Target Sequences with Motif	% of Background Sequences with Motif
1		HIF-1b(HLH)/T47D-HIF1b-ChIP-Seq(GSE59937)	1e-103	0.0000	8.78%	7.86%
2		Arnt:Ahr(bHLH)/MCF7-Arnt-ChIP-Seq(Lo_et_al.)	1e-63	0.0000	4.28%	3.77%
3		HIF-1a(bHLH)/MCF7-HIF1a-ChIP-Seq(GSE28352)	1e-40	0.0000	1.96%	1.69%
4		HIF2a(bHLH)/785_O-HIF2a-ChIP-Seq(GSE34871)	1e-36	0.0000	2.82%	2.51%
5		n-Myc(bHLH)/mES-nMyc-ChIP-Seq(GSE11431)	1e-14	0.0000	3.57%	3.35%
6		HNF6(Homeobox)/Liver-Hnf6-ChIP-Seq(ERP000394)	1e-14	0.0000	7.30%	6.99%
7		c-Myc(bHLH)/mES-cMyc-ChIP-Seq(GSE11431)	1e-10	0.0000	2.48%	2.32%
8		Cux2(Homeobox)/Liver-Cux2-ChIP-Seq(GSE35985)	1e-9	0.0000	6.14%	5.91%
9		bHLHE40(bHLH)/HepG2-BHLHE40-ChIP-Seq(GSE31477)	1e-9	0.0000	1.61%	1.49%
10		Max(bHLH)/K562-Max-ChIP-Seq(GSE31477)	1e-8	0.0000	3.79%	3.62%

B. TFBS motifs enriched in DMRs

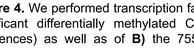
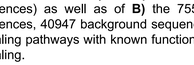
Rank	Motif	Name	p-value	q-value (Benjamini)	% of Target Sequences with Motif	% of Background Sequences with Motif
1		HIF-1b(HLH)/T47D-HIF1b-ChIP-Seq(GSE59937)	1e-3	0.0481	11.46%	9.50%
2		Arnt:Ahr(bHLH)/MCF7-Arnt-ChIP-Seq(Lo_et_al.)	1e-3	0.0496	5.84%	4.51%
3		HIF-1a(bHLH)/MCF7-HIF1a-ChIP-Seq(GSE28352)	1e-2	0.5473	2.68%	2.00%

Figure 4. We performed transcription factor binding site (TFBS) motif analysis using Homer Motif Analysis[9] of **A)** all significant differentially methylated CpGs (q<0.01, 419863 total target sequences and 403219 background sequences) as well as of **B)** the 7558 DMRs using default settings with a 200bp window (3202 total target sequences, 40947 background sequences). We found striking enrichment of binding sites of transcription factors in signaling pathways with known function in trophoblast development and response to CSE including HIF-1 and AHR signaling.

Figure 5. Top IPA biological pathways associated with genes in DMRs

A. Top Canonical Pathways

Name	p-value	Overlap
Antigen Presentation Pathway	5.97E-03	5.3% (2/38)
Aryl Hydrocarbon Receptor Signaling	9.30E-03	2.1% (3/142)
Graft-versus-Host Disease Signaling	9.39E-03	4.2% (2/48)
Autoimmune Thyroid Disease Signaling	9.77E-03	4.1% (2/49)
Human Embryonic Stem Cell Pluripotency	9.81E-03	2.1% (3/145)

B. Aryl Hydrocarbon Receptor Signaling

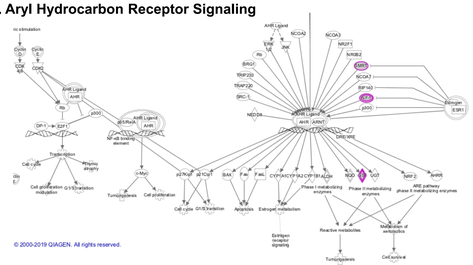


Figure5. We limited the set of associated genes (N=569) to those mapping within 10kb of a DMR (N=70) for further biological annotations using IPA and **A)** found again the AHR signaling pathway among the top enriched canonical pathways. **B)** The identified target genes mapping to the AHR pathway within 10kb of a DMR are highlighted in purple.

Discussion

Overall, 204 transcripts were differentially expressed between placenta samples with (n=18) or without (n=17) exposure to maternal CSE (p-value <0.01). As expected, *CYP1A1* expression was induced in samples with maternal CSE (Figure 2A). Transcripts for *CGB* (Figure 2B) and *ESRRB* (not shown) were also differentially expressed with respect to maternal CSE. *CGB* expresses the β-subunit of hCG. Elevated levels of hCG in maternal serum in the first and second trimesters of pregnancy have been associated with the risk of having an infant that is SGA[6]. The induction of *CGB* by components of cigarette smoke is consistent with this association and is currently under further investigation. Esrrb is a transcription factor that has been shown to play an important role in placenta development in mouse models[10] but its role in human placenta development is unknown.

Network analysis with IPA of transcripts with at least a 2-fold change in expression between samples without maternal CSE identified the upstream regulator cigarette smoke is activated in this data set in response to maternal CSE (Figure 2C). Six of the seven target genes in the dataset exhibit changes in gene expression upon exposure to maternal CSE consistent with activation (Figure 2D).

We uncovered remarkable epigenetic changes in placentas from women exposed to tobacco smoke. WGBS identified 7558 DMRs each containing >3 CpGs in a window of 200 bp and 542 DMRs with at least 5 differentially methylated CpGs. TFBS analysis demonstrated that these DMRs are enriched for binding sites of transcription factors with known function in trophoblast development and response to CSE including HIF-1 and AHR (Figure 4).

Network analysis of DNA methylation signature-associated targeted genes for regions mapping to these modules were identified by GREAT and pathway analysis by DAVID was applied. Strikingly, we consistently observed Tobacco Use Disorder and Aryl hydrocarbon signaling as the top pathways associated with gene regions exhibiting differential methylation (Figures 3 & 5).

Conclusions

Maternal CSE results in striking changes in the transcriptome and methylome of human placenta early in pregnancy. These changes provide insights into the mechanisms leading to the increased risk of SGA of infants exposed to maternal CSE *in utero*. Current studies are investigating the role of major biological pathways and target genes identified through the profiles of the transcriptome and methylome on placenta development and function.

References

1. Tong VT, et al. Trends in smoking before, during, and after pregnancy--Pregnancy Risk Assessment Monitoring System, United States, 40 sites, 2000-2010. *MMWR Surveill Summ.* 2013;62(6): 1-19.
2. Raisanen S, et al. Contribution of socioeconomic status to the risk of small for gestational age infants--a population-based study of 1,390,165 singleton live births in Finland. *Int J Equity Health.* 2013;12: 28.
3. Doherty SP, et al. Early life insult from cigarette smoke may be predictive of chronic diseases later in life. *Biomarkers.* 2009;14 Suppl 1: 97-101.
4. Vyhlidal CA, et al. Cotinine in human placenta predicts induction of gene expression in fetal tissues. *Drug Metab Dispos.* 2013;41(2): 305-311.
5. Bray NL, et al. Near-optimal probabilistic RNA-seq quantification. *Nat Biotech.* 2016 34, 525–527.
6. Goto E. Meta-regression analysis to evaluate relationships between maternal blood levels of placentation biomarkers and low delivery weight. *Int J Gynecol Obstet.* 2018 142:148-155.
7. Huang DW, et al. Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. *Nature Protoc.* 2009 4(1): 44-57.
8. Huang DW, et al. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* 2009 37(1): 1-13.
9. Heinz S, et al. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol Cell.* 2010 38(4): 576-589.
10. Latos PA, et al. Fgf and Esrrb integrate epigenetic and transcriptional networks that regulate self-renewal of trophoblast stem cells. *Nat Commun.* 2015 6:7776.

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